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Year-and-a-Half Old, Dried *Echinacea* Roots Retain Cytokine-Modulating Capabilities in an *in vitro* Human Older Adult Model of Influenza Vaccination

Original Paper

Abstract

Alcohol tinctures prepared from aged *Echinacea* roots are typically taken for preventing or treating upper respiratory infections, as they are purported to stimulate immunity in this context. The effects of long-term (> 1 year) dry storage on the capabilities of *Echinacea* spp. roots from mature individuals to modulate cytokine production are unknown. Using an older human adult model of influenza vaccination, we collected peripheral blood mononuclear cells from subjects 6 months post-vaccination and stimulated them *in vitro* with the two Type A influenza viruses contained in the trivalent 2004–2005 vaccine with a 50% alcohol tincture prepared from the roots of one of seven *Echinacea* species: *E. angustifolia*, *E. pallida*, *E. paradoxa*, *E. purpurea*, *E. sanguinea*, *E. simulata*, and *E. tennesseensis*. Before being processed into extracts, all roots had been stored under dry conditions for sixteen months. Cells were cultured for 48 hours; following incubation, supernatants were collected and assayed for interleukin-2, interleukin-10, and interferon-γ production, cytokines important in the immune response to viral infection. Four species (*E. angustifolia*, *E. purpurea*, *E. simulata*, *E. tennesseensis*) augmented IL-10 production, diminished IL-2 production, and had no effect on IFN-γ production. *Echinacea pallida* suppressed production of all cytokines; *E. paradoxa* and *E. sanguinea* behaved similarly, although to a lesser extent. The results from these *in vitro* bioactivity assays indicate that dried *Echinacea* roots stored for sixteen months maintain cytokine-modulating capacities. Our data support and extend previous research and indicate that tinctures from different *Echinacea* species have different patterns of immune modulation; further, they indicate that certain species may be efficacious in the immune response to viral infection.

Key words

Echinacea angustifolia · *Echinacea pallida* · *Echinacea paradoxa* · *Echinacea purpurea* · *Echinacea sanguinea* · *Echinacea simulata* · *Echinacea tennesseensis* · interleukin-2 · interleukin-10 · interferon-gamma · influenza · tincture

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Introduction

The use of *Echinacea* phytomedicinal preparations has grown in the past few decades [1], [2]. *Echinacea* has documented immunomodulatory properties and is most frequently consumed to prevent or minimize symptoms from upper respiratory infections, although scientific studies regarding its efficacy in this capacity report dissimilar findings [3]. *Echinacea* can alter patterns

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of cytokine production *in vitro* as shown previously [4], [5], and this may increase resistance to infection. Some cytokines important in response to viral infection include interleukin-2 (IL-2; enhances T-helper cell function, important in adaptive immunity against many viruses), IL-10 (regulator of cytokine synthesis with anti-inflammatory actions), and interferon-γ(IFN-γ, an antiviral cytokine). Although a well-controlled clinical trial recently demonstrated that *Echinacea angustifolia* did not minimize viral load in response to rhinovirus challenge [6], it is possible that various *Echinacea* species may alter cytokine expression in such a way as to reduce inflammation and associated symptoms.

Recently, progress has been made to better understand the effects of drying on bioactive constituents of *Echinacea* roots. Both commercial manufacturers and lay herbalists commonly employ drying processes. The effects of commercial drying regimens (i.e., high temperatures and short drying times, sometimes involving techniques such as convection drying, freeze drying, or vacuum drying) on bioactive constituents of *Echinacea* have been studied [7], [8], [9], [10], [11], [12], [13]; however, various teams report conflicting findings. It is even less clear how drying conditions such as those a lay herbalist would employ may affect bioactivity of *Echinacea* roots; however, Perry et al. [14] have reported that alkamide levels dropped 80 % when *E. purpurea* roots were stored at 24 °C for 64 weeks, a method similar to that employed by lay herbalists. Data on the effects of longer-term storage (> 1 year) are lacking. Medicinal plants may be capable of retaining their medicinal properties long after harvest, as has been recently demonstrated with an 85-year-old specimen of black cohosh (*Actaea racemosa* L.) [15].

The effects of plant age on root bioactive constituent concentrations have also been investigated. However, the results of these studies are conflicting. The roots from older plants may have increased, decreased, or similar levels of constituents compared to younger counterparts per the specific constituent [16], [17].

While numerous phytomedicinal studies of *Echinacea* spp. roots have been reported, only a handful of these reports state specifically that dried roots were employed. *In vitro* cytokine production from mouse splenocyte cultures stimulated with water and ethanol/water extracts of dried *E. purpurea* roots and leaves has been investigated. Various extracts increased production of IL-6, IL-10, MIP-1α, and TNF-α, but not IL-2, IL-12, or IFN-γ[4]. In other studies, *E. purpurea* dried root powder has shown cyclooxygenase-inhibiting properties in both *in vitro* [18] and *ex vivo* [19] models. Granulocytes from mice fed *Echinacea* (several spp.) demonstrated higher levels of stimulation both during and immediately after the feeding period as compared to granulocytes from control mice [20]. Splenic lymphocytes from mice treated with *E. purpurea* dried root powder showed greater apoptosis resistance compared to non-treated controls, suggested by down-regulation of Fas-Ag expression and up-regulating Bcl-2 expression at the cell surface [21]. In combination with roots of wild indigo (*Baptisia tinctoria* L. [Vent], Fabaceae) and shoots of white cedar (*Thuja occidentalis* L., Cupressaceae), extracts of dried *E. pallida* and *E. purpurea* roots lowered mortality, increased mean survival time, reduced lung consolidation, and reduced lung viral titer in mice infected with influenza Type A [22].

Despite these data, it is difficult to compare the results from different researchers because several important variables were not reported, including: (a) age of plant at time of harvest; (b) length of drying time; (c) drying and storage conditions; (d) accession and origin of plant materials used; and (e) plant handling conditions (i.e., was the plant kept intact, portioned, chopped, etc.?).

The aim of the present study was to investigate the effects of sixteen months of dry storage on the immunomodulatory properties of roots from defined accessions of seven *Echinacea* species by using an older human adult model of influenza vaccination. Our hypothesis was that *Echinacea* roots stored under dry conditions for sixteen months and then processed into alcohol tinctures would retain cytokine-modulating capabilities, although these effects would vary by species. To test this hypothesis, we isolated peripheral blood mononuclear cells from older humans (> 64 years of age) who had been vaccinated with the trivalent influenza vaccine 6 months prior to this study. Cells were incubated *in vitro* for 48 hours with *Echinacea* tinctures or control, along with influenza antigen. We tested supernatants for the synthesis of IL-2, IL-10, and IFN-γ, cytokines known to be important in the immune response to upper respiratory infections such as influenza [23].

Materials and Methods

Plant collection, drying, and storage

All plant material used in this study was harvested in December 2003 from the North Central Regional Plant Introduction Station in Ames, Iowa. Every attempt was made to keep root bundles intact upon excavation; further, all aerial parts were left attached. The following species were collected and are given with their respective accession identifiers as well as the abbreviations by which they and the corresponding tinctures will be referred to in this publication: *E. angustifolia* var. *strigosa* (**ANG**; PI631302A), *E. pallida* (**PAL**; PI631275), *E. paradoxa* var. *neglecta* (**PAR**; PI631263), *E. purpurea* (**PUR**; unknown parentage), *E. sanguinea* (**SAN**; PI631257C), *E. simulata* (**SIM**; PI631304A), and *E. tennesseensis* (**TEN**; PI631250C). Voucher specimens for PUR, SAN, and TEN were deposited at Ada Hayden Herbarium, Ames, IA (ISC 435 975, 434 027, and 435 980, respectively). Vouchers for ANG, PAL, and SIM were prepared by K. McKeown (McKeown #97 004, 97 007, 97 002, respectively). All collections are available through the Station; images and respective data may be found at http://www.ars-grin.gov/npgs.

Whole plants were taken to the laboratory for temporary storage before being moved to a climate-controlled facility for sixteen months of storage. This room was held at 25.5 – 26.5 °C and 38 % relative humidity during the study as determined by a separate temperature hygrometer. Plants were hung upside down during this time. No special plant preparatory or drying procedures were used.

Extract preparation

Following storage, plants were removed to the laboratory where ethanol tinctures consisting of 50 % ethanol and 50 % sterile water were prepared from roots. In all cases, plant cross-sections and/or shavings were produced using a surgical scalpel and com-

bined with solvent as 1 part plant, 9 parts solvent. Preparations were steeped at room temperature for 20 minutes on a horizontal agitator before being filtered through sterilized tulle and stored at –20 °C until use.

High-performance liquid chromatography

Phytochemical analysis was performed to detect alkamides and caffeic acid derivatives in the *Echinacea* extracts with the use of high-performance liquid chromatography (HPLC). Before analysis, into 160 μL of *Echinacea* extracts, 15 μL (1 mg mL^{-1}) *N*-isobutylundeca-2-ene-8,10-diynamide ($C_{15}H_{21}O_2$) and 15 μL (1 mg mL^{-1}) 3,5-dimethoxy-4-hydroxycinnamic acid ($C_{11}H_{12}O_5$) were added as internal standards for quantification of lipophilic metabolites and hydrophilic metabolites, respectively. Fifteen microliters of each sample were injected into a Beckman Coulter HPLC with a 508 autosampler, 126 pump control and 168 UV-photodiode array detector controlled by 32karat^{TM} software (Version 5.0), and a YMC-Pack ODS-AM RP C18 (250×4.6 mm, 5 μm) analytical column (Waters; Bedford, MA, USA). The solvent system for lipophilic constituents was acetonitrile/H_2O at a flow rate of 1.0 mL/min following a linear gradient of 40–80 % acetonitrile over 45 min. The solvent system for hydrophilic constituents consisted of acetonitrile/H_2O and 0.01 % formic acid, at a flow rate of 1.0 mL/min following a linear gradient of 10–35 % acetonitrile over 25 min. Online UV spectra were collected between 190 and 400 nm.

For compound identification, alkamides **8**/**9**, cichoric acid, echinacoside, caftaric acid and cynarin were purchased from Phytolab (Vestenbergsgreuth, Germany)**;** chlorogenic acid was purchased from Sigma Aldrich (St. Louis, MO, USA); alkamides **8**, **10**, **11**, **12**, **13**, and **14** and ketone **22** were synthesized by Dr. George Kraus, Department of Chemistry, Iowa State University [24], [25]. In the absence of standards, alkamides **1**, **2**, **3**, **4**, **5**, **7** and ketone **24** were identified by HPLC fractionation coupled with GC-MS analysis. Phytochemicals were quantified based on the internal standard with the limit of HPLC detection at approximately 0.02 μg/mL.

Human subjects and PBMC isolation

All procedures involving human subjects were approved by the Institutional Review Board (ethics commission) at Iowa State University and Des Moines University of Osteopathic Medicine. Forty subjects were recruited. Exclusion/inclusion criteria were dependent on age, health status, and medication and supplement use. The minimal age of subjects was 64. Subjects reporting cancer within the last 5 years, or autoimmune disorders or other disorders likely to impact on immune variables, were excluded. Individuals treated with medications that might significantly alter immune variables (i.e., oral corticosteroids) or medications for treating anxiety/depression were excluded. In addition, subjects taking dietary supplements thought to alter immune responses were excluded. In October 2004, participants received the trivalent influenza vaccine containing A/New Caledonia/20/99 (H1N1), A/Wyoming/03/2003 (H3N2), and B/Jiangsu/10/2003 (Aventis Pasteur; Swiftwater, PA, USA). Six months postvaccination (April 2005), subjects returned to the laboratory for a blood draw to be used for testing the *Echinacea* extracts. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque plus (Amersham Pharmacopia Biotech; Piscataway, NJ, UAS) gradient centrifugation, counted manually using a he-

macytometer, and adjusted to a concentration of 1.0×10^6 cells/mL in AIM-V media (GIBCO/Invitrogen; Carlsbad, CA, USA).

Vaccine efficacy

To determine whether the vaccine elicited an immunological response in subjects, hemagluttination inhibition (HI) assays as described elsewhere by Kohut et al. [26] were performed. Sera were collected pre- and four weeks post-vaccination, and HI titer (antibody response) was measured for the Influenza A/New Caledonia/20/99 (H1N1) and Influenza A/Wyoming/03/2003 (H3N2) viruses contained in the vaccine.

Influenza-specific activation of PBMCs

1.0×10^6 cells were plated per well in 24-well Costar plates. Each well received either 50 μL of one of the *Echinacea* preparations diluted 1 : 12.5 or a solvent vehicle control containing an equal volume of ethanol as the extract. The ethanol for the solvent vehicle control was diluted in AIM-V media. This extract concentration was selected based on preliminary studies of cell ethanol tolerance (data not shown). Wells received 10 HA units/mL of either the Influenza A/New Caledonia/20/99 (H1N1) virus or the Influenza A/Wyoming/03/2003 (H3N2) virus identical to that contained in the vaccine to stimulate virus-specific cytokine production. Based on several previous studies, the wells that do not contain virus typically have cytokine levels ranging from undetectable up to 10–30 pg/mL at peak time points of *in vitro* culture. The cytokine levels in wells containing virus are typically 10- to 100-fold greater than the cytokine level in wells containing media or vehicle alone, suggesting that the response detected is antigen-specific. The *Echinacea* preparations were added to culture wells at the same time that virus was added. Control wells received virus identical to *Echinacea*-treated wells, but received the solvent vehicle control instead of an *Echinacea* preparation.

Cytokine assays

Cells were incubated for 48 hours at 37 °C, 5.0 % carbon dioxide in humidified conditions for the cytokine assay. Following incubation, the supernatants were harvested and stored at –20 °C until used in cytokine quantification assays. Cytokines [interleukin-2 (IL-2), IL-10, and interferon-γ (IFN-γ)] were quantified by ELISA (BD Biosciences Pharmingen; San Diego, CA, USA).

Endotoxin determination

Measures were taken to minimize endotoxin contamination. All glassware was baked at 180 °C for 24 hours prior to use. Sterile water was obtained from the manufacturer labeled for human medical use. Endotoxin levels were determined from both the sterile water (0 endotoxin units [EU]/mL) and from all final extracts by using Bio-Whitaker QCL 1000 kits (Cambridge, MA, USA). Levels (in EU/mL) from the extract stocks were as follows: ANG 8.925, PAL 8.553, PAR 9.817, PUR 8.999, SAN 9.222, SIM 8.255, TEN 9.743.

To experimentally determine whether the endotoxin levels seen in the extracts influenced our immune outcomes, we isolated PBMC from four healthy, young adults according to the methods described above. Cells were cultured for 24 hours in AIM-V media with 50 μL of stock concentrations of *Escherichia coli* endotoxin at 10 EU/mL or 100 EU/mL or sterile water (0 EU/mL). The 10 EU/mL stock represented an endotoxin level > 12.5 ×higher than our

Echinacea diluted extract with the highest endotoxin level (PAR), and the 100 EU/mL stock represented an endotoxin level known to be immunostimulatory. For the endotoxin assay, supernatants were collected and cytokines [IL-2, IL-10, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α)] quantified by ELISA (BD Biosciences Pharmingen).

Statistical analyses

A two-way ANOVA [species × virus type] was used to compare differences in cytokine production by using SPSS (Chicago, IL, USA) software. One-way ANOVA was used to determine the effects of different endotoxin doses on cytokine production. Statistical significance was defined as α = 0.05.

Results

Extracts prepared by using methods resembling those of lay herbalists may harbor endotoxin from contaminating bacterial populations growing on the plant, and endotoxin is a known mitogen *in vitro*. To determine experimentally if endotoxin levels in our extracts may be influencing immune outcomes, we tested stock concentrations of *E. coli* endotoxin against human PBMC. Results are shown in Fig. 1. Cytokine production from cells receiving the diluted stock of 10 EU/mL was not significantly different from control (0 EU/mL) for IL-1β (p = 0.995), IL-10 (p = 0.973), and TNF-α (p = 0.98); however, cells receiving the diluted stock of 100 EU/mL produced higher levels of cytokine as compared to control for IL-1β (p = 0.068), IL-10 (p = 0.044), and TNF-α (p = 0.005). No differences were observed for IL-2, which is expected, as this is primarily a T-cell-produced cytokine with little responsiveness to endotoxin [27].

The phytochemical analysis of our extracts for alkamides, ketones, and caffeic acid derivatives is presented in Table 1 and Fig. 1S in the Supporting Information. Extracts from ANG, PUR, and SAN had the highest concentrations of alkamides. PAL, PAR, and SIM had the highest concentrations of ketones. Contents of caffeic acid derivatives differed by specific molecule. Overall, PUR had the highest total concentration of caffeic acid derivatives, and PAL had the greatest diversity of molecules.

Hemagluttination inhibition assays for vaccine efficacy indicated that subjects responded to the vaccination. Antibody titers for Influenza A/Wyoming/03/2003 (H3N2) were 6.9 ± 1.5 pre-vaccination compared to 8.0 ± 1.8 post-vaccination (*p* < 0.001). Antibody titers for Influenza A/New Caledonia/20/99 (H1N1) increased, but were not significantly different (6.38 ± 1.0 pre-vaccination, 6.43 ± 1.2 post-vaccination), likely due to the presence of this same H1N1 antigen in several previous years' influenza vaccines, as other researchers have also shown in this age group [28].

Results for interleukin-10 production are given in Fig. 2. A significant main effect of plant species was observed (p < 0.001) as well as a plant species by virus interaction (p = 0.038). Follow-up analyses were conducted separating results from the two viruses. For Influenza A/New Caledonia/20/99 (H1N1) alone, a significant effect of plant species remained (p = 0.001). Four extracts stimulated significant increases in cell production of IL-10 compared to the control condition containing no *Echinacea* (Fig. 2A): ANG (p < 0.001), PUR (p < 0.001), SIM (p = 0.001), and TEN (p = 0.002). PAL significantly decreased IL-10 production (Fig. 2A; p = 0.005). A similar pattern of results was found for the H3N2 virus. For Influenza A/Wyoming/03/2003 (H3N2) alone, a significant effect of plant species remained (p < 0.001).

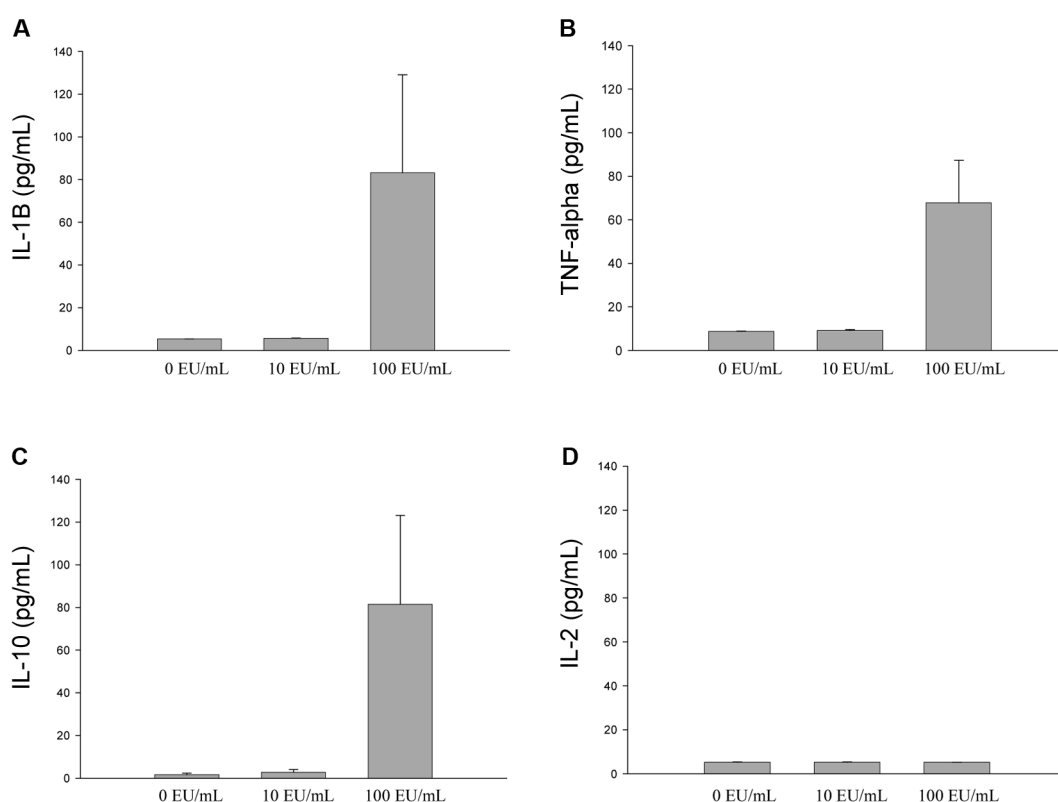Fig. 1 Cytokine production (in pg/mL) by PBMC stimulated with various levels of endotoxin: (**A**) IL-1β, (**B**) TNF-α, (**C**) IL-10, (**D**) IL-2. Bars represent standard errors.

Table **1** Phytochemical profile of *Echinacea* extracts used in this paper as determined by HPLC

Compound	ANG	PAL	PAR	PUR	SAN	SIM	TEN
amide **1**	ND	ND	ND	0.0456	0.0718	ND	ND
amide **2**	0.0059	0.0008	ND	0.0159	0.0175	ND	ND
amide **3**	0.0059	0.0087	ND	0.0310	0.2011	ND	ND
amide **4**	0.0059	ND	ND	ND	0.0213	ND	ND
amide **5**	0.0124	ND	ND	0.0156	ND	ND	ND
amide **7**	0.0000	ND	ND	0.0092	0.0559	ND	ND
amide **8**	0.2798	ND	ND	0.2912	1.3138	ND	ND
amide **9**	0.0343	ND	ND	0.0396	0.3387	ND	ND
amide **10**	0.0368	ND	ND	0.0102	0.0378	ND	ND
amide **11**	0.0486	ND	ND	0.0106	0.0286	ND	ND
amide **12**	0.0371	ND	ND	ND	0.2782	ND	0.0024
amide **13**	0.0449	ND	ND	ND	0.1444	ND	0.0098
amide **14**	0.0141	ND	ND	ND	0.4444	ND	0.0085
ketone **22**	ND	0.0540	0.0029	ND	ND	0.0148	ND
ketone **24**	ND	0.1668	0.0301	ND	ND	0.0093	ND
caftaric acid	ND	0.0535	ND	0.1568	0.0113	0.0359	ND
chlorogenic acid	0.0137	0.0079	0.0266	0.0157	0.0509	0.0069	0.0088
cichoric acid	ND	0.0141	ND	1.0147	ND	ND	ND
cynarin	0.0223	ND	ND	ND	ND	ND	0.0094
echinacoside	0.0310	0.0885	0.1472	ND	ND	0.0476	0.0436

Species abbreviations: ANG (*E. angustifolia* var. *strigosa*), PAL (*E. pallida*), PAR (*E. paradoxa* var. *neglecta*), PUR (*E. purpurea*), SAN (*E. sanguinea*), SIM (*E. simulata*), and TEN (*E. tennesseensis*).
ND = Not detectable.

Extracts from the same four species stimulated increases in IL-10 production (Fig. **2B**): ANG (p < 0.001), PUR (p = 0.002), SIM (p = 0.001), and TEN (p = 0.010). PAL again significantly decreased IL-10 production (p < 0.001).

Results for interleukin-2 production are shown in Fig. **3**. A significant main effect of plant species was found (p < 0.001). All seven extracts induced statistically significant (all p < 0.001) reductions in IL-2 production compared to control condition containing no *Echinacea*.

Results for interferon-γ production are displayed in Fig. **4**. A significant main effect of plant species was observed (p < 0.001). No extracts significantly augmented IFN-γ production. However, three extracts significantly diminished IFN-γ synthesis: PAL (p < 0.001), PAR (p < 0.001), and SAN (p = 0.003).

Discussion

In this study, we employed an *in vitro* human older adult model of influenza vaccination to assess the bioactivity of *Echinacea* roots stored dry for sixteen months, specifically focusing on their ability to modulate patterns of cytokine expression in response to antigen challenge. This model had several virtues that made it readily amenable to the questions being addressed in this investigation. *Echinacea* extracts are typically taken for the prevention or treatment of upper respiratory infections, such as influenza [29]. The model chosen here allowed us to test the potential efficacy of *Echinacea* extracts in an *in vitro* context of viral infection using human PBMC. The elderly are one segment of the population that typically consumes *Echinacea* preparations in an at-

tempt to "boost" their immunity [30]. Influenza vaccine efficacy rates in geriatric populations are typically poorer than those of young adult controls, and influenza infection is a major cause of morbidity and mortality in this group [31]. Thus, results from this study may provide information on the potential feasibility of using *Echinacea* preparations to improve influenza vaccine efficacy rates in older adults. In addition, a better understanding of how *Echinacea* may modulate cytokines in response to influenza virus challenge *in vitro* may be a first step in understanding the potential role of *Echinacea* during an influenza infection *in vivo*.

The effects of endotoxin in our extracts were explored experimentally (Fig. **1**). Cells stimulated with endotoxin levels somewhat higher than levels in the extracts behaved no differently than controls that contained no endotoxin. These findings suggest that endotoxin levels in our *Echinacea* extracts did not influence the immune outcomes reported here.

Results for our cytokine assays (Figs. **2–4**) are summarized in Table **2**. As is clear from these figures, even after sixteen months of dry storage, *Echinacea* roots maintained cytokine-modulating capabilities.

We saw contrasting effects of preparations made from different *Echinacea* species on the production of interleukins. In general, *Echinacea* preparations tended to have no effect or increased production of IL-10 (Fig. **2**; Table **2**). In contrast, all tinctures decreased production of IL-2 (Fig. **3**; Table **2**). Table **1** does not suggest any single molecule or class of molecules that may be responsible for the IL-10 activities seen in Table **2**. According to Table **1**, amides and caffeic acid derivatives (but not ketones) were present in all species to varying extents. Chlorogenic acid
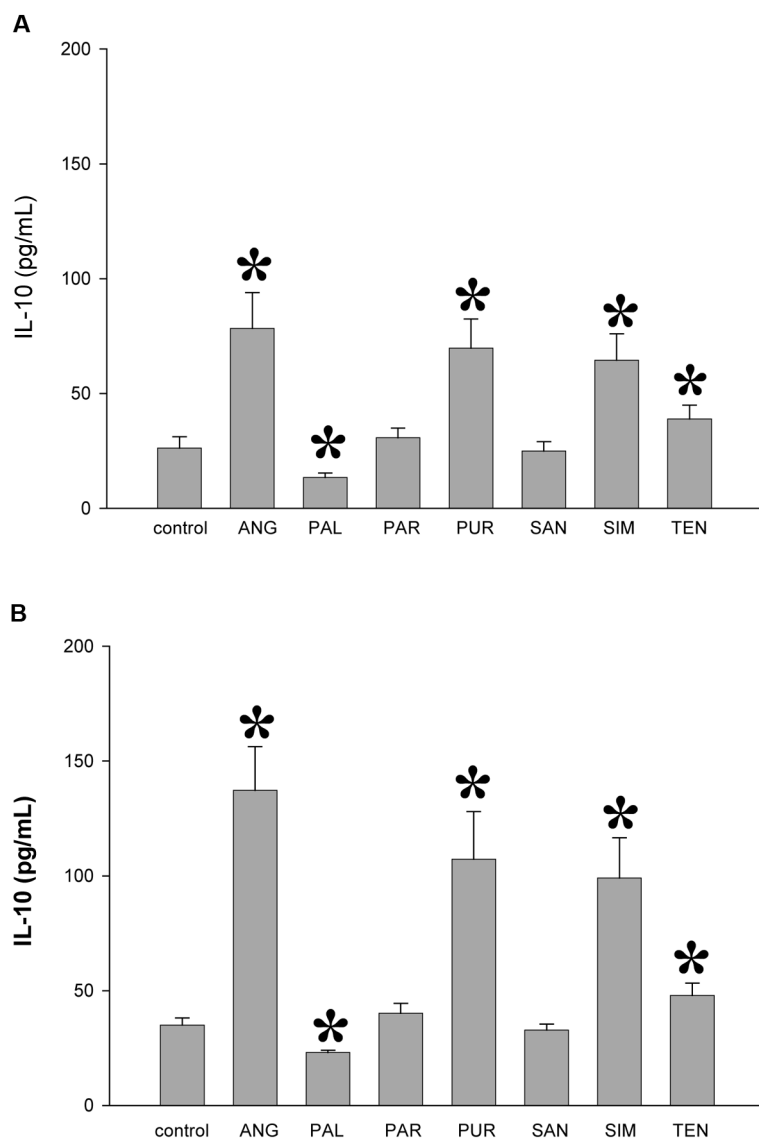
A

B

Fig. 2 IL-10 production (in pg/mL) by *Echinacea* tinctures under H1N1 (**A**), and H3N2 (**B**) stimulation. Bars represent standard errors. Note the direction of significance. Species abbreviations: ANG (*E. angustifolia* var. *strigosa*), PAL (*E. pallida*), PAR (*E. paradoxa* var. *neglecta*), PUR (*E. purpurea*), SAN (*E. sanguinea*), SIM (*E. simulata*), and TEN (*E. tennesseensis*).

was the only molecule detectable in extracts from all seven species. It is possible that multiple alkamides or multiple caffeic acid derivatives may be responsible for this activity. One possible explanation for these results is that compounds in *Echinacea* may promote a down-regulation of the immune response following viral infection (e.g., by producing more IL-10 and less IL-2). This explanation may also support those studies finding a beneficial effect of *Echinacea* during viral infection [32].

With respect to our findings, *Echinacea* did not appear to enhance IFN-γ (Fig. **4**; Table **2**). Some species inhibited IFN-γ, suggesting that these species may not be efficacious in stimulating antibody production if consumed at the time of vaccination. No single molecule or group of molecules from Table **1** was unique to these three species, making it unclear which constituent(s) are responsible for these activities. Our results suggest that *Echinacea* preparations made in the manner employed here may not have a large effect on the production of IFN-γ. Though IFN-γ response following influenza vaccination is reduced in older adults compared to younger counterparts [33], researchers have shown that increases in IFN-γ correlate with increases in antibody titer in vaccinated elderly [34]; similarly, a poor Th1 (IFN-γ) response to influenza vaccine is correlated with reduced antibody response to the vaccine [35]. Therefore, our findings based on the IFN-γ response may suggest that *Echinacea* administered at the time of immunization is not likely to increase antibody response to the vaccine, but direct evidence regarding this possibility is not available from our study. In older adults, the vaccine does not provide complete protection. In the event of influenza infection, cell-mediated immune responses become important clearing virus infection. Cell-mediated responses may be measured by examining proliferative response of PBMC to appropriate antigen. Studies have shown that the proliferative response of PBMC is correlated with influenza-stimulated production of IL-10 in older adults [23]. IL-10 also appears to be necessary for the development of specific antibody subclasses in response to influenza vaccine [36]. Based on our results, it is possible that the *Echinacea*-associated increase in IL-10 may direct antibody subclass development if *Echinacea* had been administered at the time of vaccination and/or improve response to infection by promoting proliferation of appropriate cell populations. However, clinical trials will be necessary to determine whether *Echinacea* may alter vaccine efficacy and/or modulate immune responses to influenza virus infection.
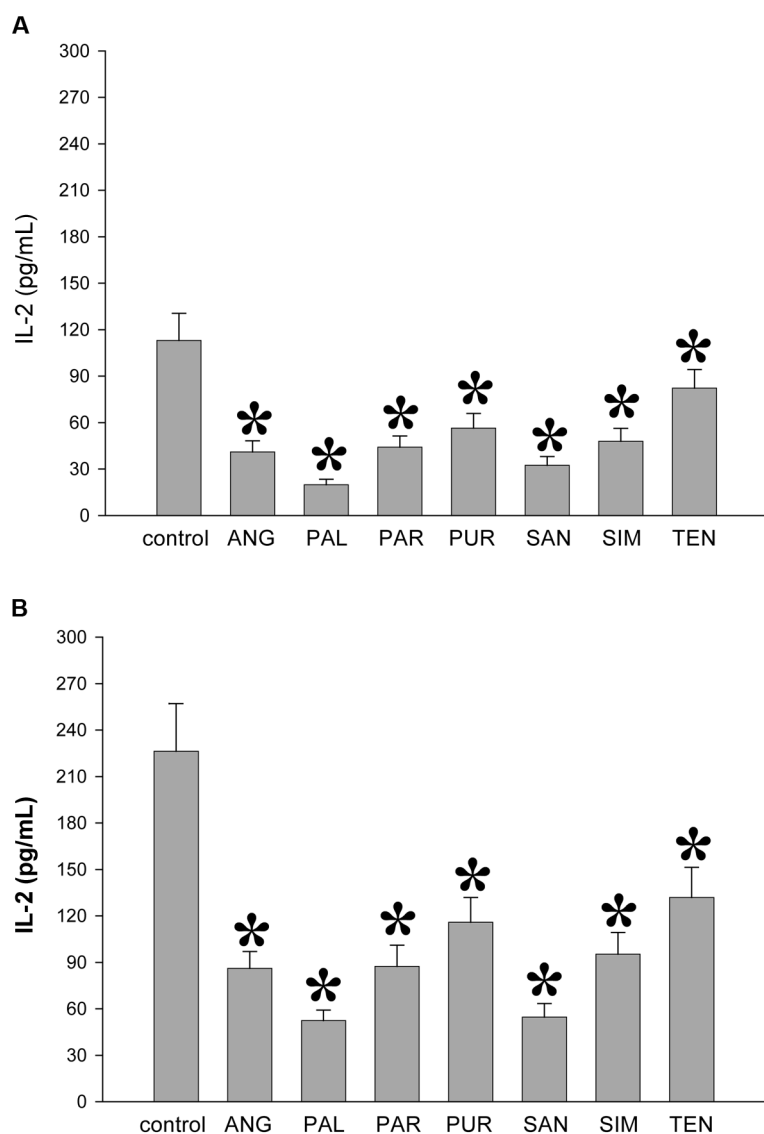
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Seven species of *Echinacea* were employed under the same experimental conditions, allowing us to make comparisons across taxa. The most consistent responses were seen from cells stimulated with PAL: in all cases, PAL induced decreases in cytokine production (Table **2**). Extracts from two other species, PAR and SAN, also decreased production of IL-2 and IFN-γ, but not IL-10. ANG, PUR, SIM, and TEN augmented production of IL-10 and diminished production of IL-2; no effects were seen on IFN-γ. These results imply that not all *Echinacea* species influence cytokine expression in similar fashions, supporting previous results from our lab [5] as well as others [37], [38], [39]. More specifically, extracts from certain species, such as PAL, PAR, and SAN, tend to show repressive effects regardless of the cytokine being assayed, whereas others, such as ANG, PUR, SIM, and perhaps TEN, stimulate cytokine-specific responses. Intriguingly, the three species employed for commercial preparations are *E. angustifolia*, *E. pallida*, and *E. purpurea*, alone or in various combinations [3], [40]. Our results may indicate that these three species do not have similar effects, and may warrant a re-assessment of which species are included in commercial preparations and in what particular combinations.

In conclusion, the results from this study suggest that *Echinacea* roots stored dry for sixteen months maintain cytokine-modulating capabilities. Additionally, they suggest that different *Echinacea* species may act on cytokine expression in different fashions. Whether or not *Echinacea* extracts may have utility as influenza vaccine boosters and/or a beneficial role during actual influenza infection is not addressed directly by this study; however, our *in vitro* findings do suggest the plausibility of such an application. Finally, more studies are needed to examine relationships between the phytochemical composition of extracts and extract fractions and immunomodulatory activities of these materials to identify possible bioactive molecules and their associated effects.
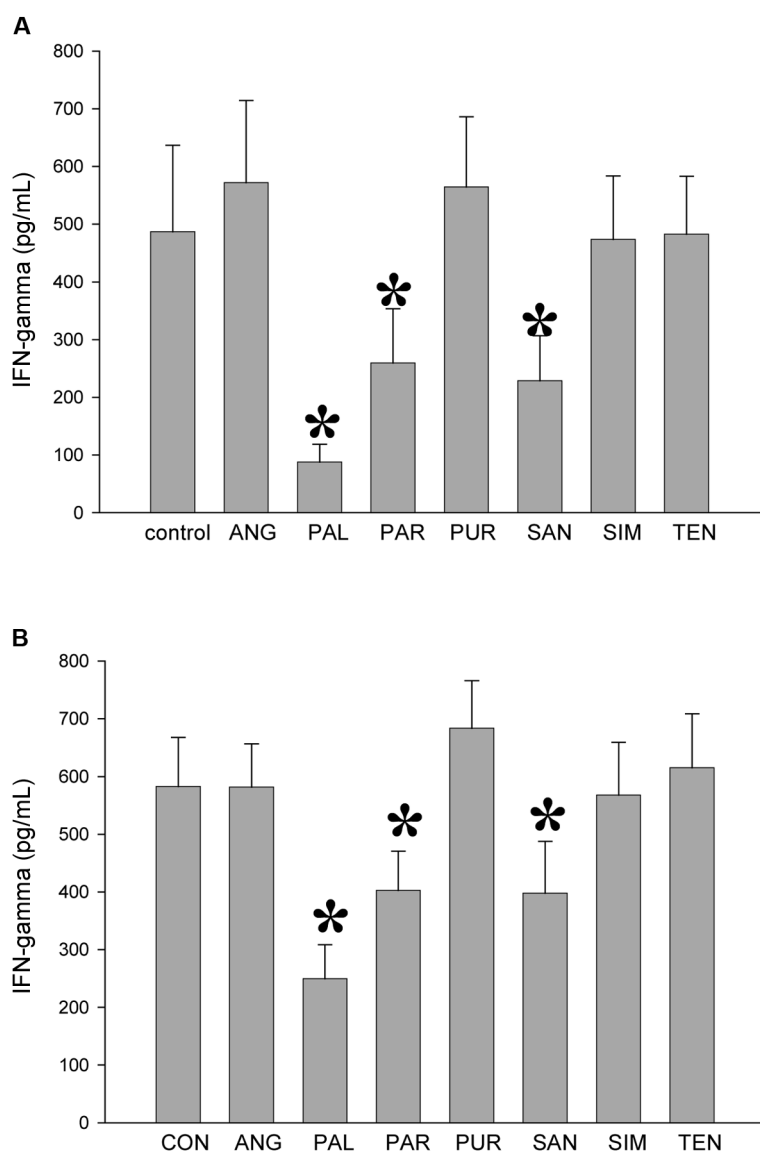
Acknowledgements

A

B

Fig. 4 IFN-γ production (in pg/mL) by *Echinacea* tinctures under HIN1 (**A**), and H3N2 (**B**) stimulation. Bars represent standard errors. Species abbreviations: ANG (*E. angustifolia* var. *strigosa*), PAL (*E. pallida*), PAR (*E. paradoxa* var. *neglecta*), PUR (*E. purpurea*), SAN (*E. sanguinea*), SIM (*E. simulata*), and TEN (*E. tennesseensis*).

Table 2 Summary of significant effects of *Echinacea* tinctures on cytokines. Arrows indicate direction of effect

	IL-2	IL-10	IFN-γ
ANG	↓	↑	
PAL	↓	↓	↓
PAR	↓		↓
PUR	↓	↑	
SAN	↓		↓
SIM	↓	↑	
TEN	↓	↑	

Species abbreviations: ANG (*E. angustifolia* var. *strigosa*), PAL (*E. pallida*), PAR (*E. paradoxa* var. *neglecta*), PUR (*E. purpurea*), SAN (*E. sanguinea*), SIM (*E. simulata*), and TEN (*E. tennesseensis*).

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els of extracts and supplied the purified endotoxin used in the experiments. Dustin A. McCann ran the ELISAs on the endotoxin experiment.

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